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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 7/10, 13/00, 15/10 A61K 39/36, C12N 15/29, 15/62 C12P 21/08, G01N 33/68	A1	(11) International Publication Number: WO 92/16554
		(43) International Publication Date: 1 October 1992 (01.10.92)

(21) International Application Number: PCT/AU92/00108

(22) International Filing Date: 13 March 1992 (13.03.92)

(30) Priority data:
PK 5084 14 March 1991 (14.03.91) AU

(71) Applicant *(for all designated States except US)*: THE UNIVERSITY OF MELBOURNE [AU/AU]; Grattan Street, Parkville, VIC 3052 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : KNOX, Robert, Bruce [AU/AU]; 9 Riverview Road, North Balwyn, VIC 3104 (AU). SINGH, Mohan, Bir [AU/AU]; 7 Lloyd Court, Templestowe, VIC 3106 (AU). SMITH, Penelope, Mary [AU/AU]; 80 Best Street, North Fitzroy, VIC 3052 (AU).

(74) Agents: SLATTERY, John, M. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: PROTEIN ALLERGENS OF THE SPECIES CYNODON DACTYLON

(57) Abstract

The present invention provides nucleic acid sequences coding for protein allergens of the species *Cynodon dactylon*, or at least one fragment thereof or the functional equivalent of such nucleic acid sequences. The present invention also provides expression vectors comprising such nucleic acid sequences and host cells transformed therewith. The present invention further provides isolated protein allergens of Bermuda grass pollen or fragments thereof. Isolated protein allergens of Bermuda grass pollen or antigenic or allergenic fragments thereof are useful for diagnosing and treating sensitivity in an individual to Bermuda grass pollen allergens.

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Protein Allergens of the Species Cynodon Dactylon

Background of the Invention:

Bermuda grass (*Cynodon dactylon*) is an important source of pollen allergens in many areas of the world, especially in tropical and sub-tropical climates. These allergens have been studied by a number of means including IgE immunoblotting (Ford D., and Baldo, B.A. *J. Allergy Clin. Immunol.* 79: 711-720 (1987); Shen H.D., et al., *Clin. Allergy* 18: 401-409 (1988), column chromatography (Orren, A., and Dowdle, *S. Afr. Med. J.* 51: 586 (1977); Matthiesen et al., *J. Allergy Clin. Immunol.* 81: 266 (Ab) (1988), and immunoelectrophoresis (Matthiesen et al., *supra*, 1988).

The presence of Bermuda grass pollen allergens in the environment causes hayfever and seasonal asthma in many individuals and continues to have significant morbidity and socio-economic impact on Western communities. While the available spectrum of drugs, including anti-histamines and steroids, have resulted in improvement in the treatment of allergic disease, they do have unfortunate side-effects associated with long term usage. Because of these problems, renewed interest has been shown in the immunotherapy of allergic disease. Immunotherapy involves the injection of potent allergen extracts to desensitize patients against allergic reactions (Bousquet, J. and Michel, F.B., *Allergy and Clin Immol. News* 1: 7-10 (1989). Unfortunately, the pollen preparations used as allergens are polyvalent and of poor quality. Consequently, concentrations of crude extracts used are frequently high and may trigger potentially lethal systemic reactions, including anaphylaxis. The product expressed from the cloned gene, fragments thereof, or synthetic peptides based on the sequence of the allergens provide a safer medium for therapy since they can be quality controlled, characterized and standardized, and they optionally do not bind IgE. Thus, identification and isolation of the protein allergens of Bermuda grass pollen would be useful in developing therapeutic and diagnostic agents to treat and diagnose sensitivity in individuals to Bermuda grass pollen allergens.

Summary of the Invention

The present invention provides nucleic acid sequences coding for protein allergens of the species *Cynodon dactylon*, or at least one fragment thereof or the functional equivalent of such nucleic acid sequences. The present invention also provides expression vectors comprising such nucleic acid sequences and host cells transformed therewith. The present invention further provides isolated protein allergens of Bermuda grass pollen or fragments thereof. Isolated protein allergens of Bermuda grass pollen or antigenic or allergenic fragments thereof are useful for diagnosing and treating sensitivity in an individual to Bermuda grass pollen allergens.

Brief Description of the Figures

Fig. 1 shows a Western blot of IgE binding to Bermuda grass pollen allergens separated by SDS-PAGE.

Fig. 2 shows IgE binding to fusion proteins on plaque lifts of cDNA clones B1, B2 and B4 of *Cynodon dactylon*

Fig. 3 is the nucleic acid sequence and deduced amino acid sequence of clone B1 of *Cynodon dactylon*

Fig. 4 shows a Western blot of Bermuda grass pollen proteins separated by SDS-PAGE and probed with sera from allergic individuals or with affinity purified IgE specifically reactive with a clone B1 fusion protein.

Fig. 5 is the nucleic acid sequence and deduced amino acid sequence of clone B4 of *Cynodon dactylon*

Fig. 6 shows the nucleotide sequence homology between clones B1 (*Cyn d*$B1) and B4 of (*Cyn d*$B4) of *Cynodon dactylon*. The first and last 6 nucleotides of each sequence represents the EcoR1 restriction sites used for cloning.

Fig. 7 shows the deduced amino acid sequence homology between clones B1 (*Cyn d*$B1) and B4 (*Cyn d*$B4) of *Cynodon dactylon*

Fig. 8 is the nucleic acid sequence and deduced amino acid sequence of clone B2 of *Cynodon dactylon*

Fig. 9 shows the deduced amino acid sequence homology between clone B2 and polygalacturonase from *Lycopersicon esculentum* (tomato).

Fig. 10 shows a Western blot of Bermuda grass pollen proteins separated by SDS-PAGE and probed with polyclonal antibodies raised against a clone B2 fusion protein.

Detailed Description of the Invention

The present invention provides nucleic acid sequences coding for protein allergens of the species *Cynodon dactylon*. The nucleic acid sequences coding for *Cynodon dactylon* allergens preferably comprise the nucleic acid sequence of Clone B1, Clone B4, and Clone B2. Nucleic acids coding for Bermuda grass pollen allergens may be obtained from any part of *Cynodon dactylon* plants. Nucleic acids encoding Bermuda grass pollen allergens may also be obtained from genomic DNA. The nucleic acids coding for protein allergens of the present invention may be obtained using the method disclosed herein or any other suitable technique for isolation and cloning of genes. The nucleic acids of the invention may be DNA or RNA.

Preferred nucleic acid sequences of the present invention comprise the nucleic acid sequences of Clone B1, as shown in Fig. 3, Clone B4, as shown in Fig. 5, and Clone B2, as shown in Fig. 8. The nucleic acid sequence and deduced amino acid sequence of Clone B1 is shown in Fig. 3. Nucleotides 1 through 213 comprise the open reading frame for Clone B1 and encode 71 amino acids. This clone encodes a partial sequence of a Bermuda grass pollen allergen. The reading frame was determined by direct nucleotide sequencing of the junction between the original pGEX-1 clone and the B1 insert. Nucleotides 214 through 468 comprise the 3' untranslated region of this clone. The nucleic acid sequence and predicted amino acid sequence for the clone B4 is shown in Fig. 5. Nucleotides 1 through 219 comprise the open reading frame for this clone and encode 73 amino acids. Clone B4 encodes a partial sequence of a Bermuda grass pollen allergen. The reading frame was determined by direct nucleotide sequencing of the junction between the original pGEX-1 clone and the B4 insert. Nucleotides 220 through 429 comprise the 3' untranslated region of this clone.

It was determined that the cDNA insert of clones B1 and B4 encode partial sequences of related protein allergens of *Cynodon dactylon*. The nucleotide homology between clones B1 and B4 is shown in Fig. 6. Clone B4 has a six nucleotide insert relative to clone B1 in the coding sequence. This nucleotide homology was demonstrated using software contained in PCGENE (Intelligenetics, Mountain View, CA). The deduced amino acid homology between clones B1 and B4 has also been determined (See Fig. 7). The deduced amino asid sequence of clone B4 has two more amino acids than the deduced amino acid sequence of clone B1. Otherwise, the amino acid sequences are identical. The amino acid homology was demonstrated using software contained in PCGENE (Intelligenetics, Mountain View, CA).

The nucleic acid sequence and deduced amino acid sequence for clone B2 is shown in Fig. 8. Nucleotides 1 through 741 form a long open reading frame that encodes 247 amino acids. There are 4 potential N-glycosylation sites with the consensus N-X-S/T sequence: amino acids 5-7 (N-A-T), amino acids 55-57 (N-V-T), amino acids 185-187 (N-I-T) and amino acids 223-225 (N-K-T). Clone B2 encodes a partial sequence of a Bermuda grass pollen allergen. The partial protein encoded by clone B2 was found to be related to polygalacturonase 2A precursor from *Lycopersicon esculentum* (tomato). Fig. 9 shows the homology between the deduced amino acid sequence of clone B2 and polygalacturonase 2A precursor from *L. esculentum*. The partial B2 protein sequence has 42.9% homology with polygalacturonase 2A precursor (33.2% identity, 9.7% similarity). This homology was demonstrated by scanning the Swiss-Prot protein data base with the FSTPSCAN program and using the PALIGN program. Both programs are contained in the PCGENE software package (Intelligenetics, Mountain View, CA).

Fragments of nucleic acid sequences coding for allergens of the species *Cynodon dactylon* are also within the scope of the invention. Fragments within the scope of the invention include those coding for portions of *Cynodon dactylon* which induce immune response in mammals, preferably humans, such as stimulation of minimal amounts of IgE binding of IgE; eliciting the production of

IgG and IgM antibodies; eliciting a T cell response such as T cell proliferation and/or lymphokine secretion and/or the induction of T cell anergy. The foregoing fragments of a protein allergen of the species *Cynodon dactylon* are referred to herein as antigenic fragments. Fragments within the scope of the invention also include those capable of hybridizing with a nucleic acid sequence from other plant species for use in screening protocols to detect allergens which are cross-reactive with a protein allergen of the species *Cynodon dactylon*. As used herein, a fragment of a nucleic acid sequence coding for a protein allergen of the species *Cynodon dactylon* refers to a nucleotide sequence having fewer bases than the nucleotide sequence coding for the entire amino acid sequence of a protein allergen of the species *Cynodon dactylon* and/or mature *Cynodon dactylon* protein allergen. Generally, a nucleic acid sequence encoding a fragment or fragments of protein allergens of the species *Cynodon dactylon* will be selected from the bases coding for the mature protein. However, in some instances, it may be desirable to select all or a part of a fragment or fragments from the leader sequence portion of the nucleic acid sequence of the invention. Nucleic acid sequences of the invention may also contain linker sequences, restriction endonuclease sites and other sequences useful for cloning, expression or purification of *Cynodon dactylon* allergens or fragments thereof.

The present invention provides expression vectors and host cells transformed to express nucleic acid sequences of the invention. A nucleic acid sequence encoding a Bermuda grass pollen allergen, or at least one fragment thereof can be expressed in a bacterial cell such as *E. coli*, insect cells, yeast, or mammalian cells such as Chinese hamster ovary cells (CHO). Suitable expression vectors, promoters, enhancers, and other expression control elements can be found in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, second edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. Expression in yeast, insect or mammalian cells would lead to partial or complete glycosylation of the recombinant material and formation of any inter- or intra-chain disulfide bonds, if such exist. Suitable vectors for expression in yeast include YepSec1 (Baldari et al., (1987) *Embo J.* 6: 229-234); pMFa (Kurjan and Herskowitz (1982) *Cell* 30: 933-943); and JRY88 (Schultz et al., (1987) *Gene* 54:

113-123).

For expression in *E. coli*, suitable expression vectors include pTRC (Amann et al., (1988) *Gene* 69: 301-315); pGEX (Amrad Corp., Melbourne, Australia); pMAL (N.E. Biolabs, Beverly, MA); pRIT5 (Pharmacia, Piscataway, NJ); and pSEM (Knapp et al. (1990) *BioTechniques* 8: 280-281). The use of pTRC and pET11d (Novagen, Madison, WI; Jameel et. al., *J.Virol.* 64: 3963-3966 (1991)) will lead to the expression of an unfused protein . The use of pMAL, pRIT5, pSEM, and pGEX will lead to the expression of an allergen fused to maltose E binding protein (pMAL), protein A (pRIT5), truncated β-galactosidase (pSEM) or glutathione S-transferase (pGEX). When a Bermuda grass pollen allergen or fragment thereof is expressed as a fusion protein, it is particularly advantageous to introduce an enzymatic cleavage site at the fusion junction between the carrier protein and the allergen or fragment thereof. The protein allergen or fragment thereof may then be recovered from the fusion protein through enzymatic cleavage at the enzymatic site and biochemical purification using conventional techniques for purification of proteins and peptides. Suitable enzymatic cleavage sites include those for blood clotting Factor Xa or thrombin for which the appropriate enzymes and protocols for cleavage are commercially available from, for example, Sigma Chemical Company, St. Louis, MO and N.E. Biolabs, Beverly, MA. Host cells can be transformed to express nucleic acid sequences of the invention using conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, or electroporation. Suitable methods for transforming the host cells may be found in Sambrook et al., *supra*, and other laboratory textbooks.

The present invention also provides methods for producing a Bermuda grass pollen allergen or at least one fragment thereof. According to one method, a host cell transformed with a DNA sequence encoding all or a portion of a Bermuda grass pollen allergen is cultured in an appropriate medium to produce a mixture of cells and medium containing the protein allergen or fragment thereof. The mixture is then purified to produce substantially pure protein allergen or a fragment thereof. A Bermuda grass pollen allergen and fragments thereof can be purified from cell culture medium, host cells, or both using techniques known in

the art for purifying peptides and proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis and immunopurification with antibodies specific for a Bermuda grass pollen allergen or fragments thereof. According to another method, isolated Bermuda grass pollen allergen or at least one fragment thereof can be synthesized chemically using techniques known in the art. The terms isolated and purified are used interchangeably herein and refer to peptides, protein, protein fragments, and nucleic acid sequences substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when synthesized chemically or when purified from pollen.

Fragments of a protein allergen from Bermuda grass pollen can be obtained, for example, by screening peptides synthesized from the corresponding fragment of a nucleic acid sequence of the invention coding for such peptides or synthesized chemically using techniques known in the art. Peptide fragments of the allergen may be obtained by any method known in the art such as chemical cleavage of the allergen, arbitrary division of the allergen into fragments of a desired length with no overlap of the peptides, or preferably division of the allergen into overlapping fragments of a desired length. The fragments can be tested to determine antigenicity (e.g., the ability of the fragment to induce an immune response). Such fragments are referred to herein as antigenic fragments. Fragments of Bermuda grass protein allergens which are capable of eliciting a T cell response such as stimulation (i.e., proliferation or lymphokine secretion) and/or are capable of inducing T cell anergy are particularly desirable. Fragments of protein allergens which do not bind immunoglobulin E (IgE) or bind IgE to a substantially lesser extent than the protein allergen from which the fragments are derived binds IgE are also particularly desirable. The major complications of standard immunotherapy are systemic responses such as anaphylaxis. Immunoglobulin E is a mediator of anaphylactic reactions which result from the binding and cross-linking of antigen to IgE on mast cells or basophils and the release of mediators (e.g., histamine, serotonin, eosinophil, chemotactic factors). Thus, anaphylaxis could be avoided by the use of a fragment which does not bind IgE, or if the fragment binds IgE, such binding does

not result in the release of mediators (e.g., histamine etc.) from mast cells or basophils. In addition, fragments which have minimal IgE stimulating activity are particularly desirable for therapeutic effectiveness. Minimal IgE stimulating activity refers to IgE stimulating activity which is less than the amount of IgE production stimulated by the whole Bermuda grass protein allergen.

Protein allergens from Bermuda grass pollen and preferred antigenic fragments thereof, when administered to a Bermuda grass pollen-sensitive individual, are capable of modifying the allergic response of the individual to the allergen, and preferably are capable of modifying the B cell, the T cell response or both the B cell and the T cell response of the individual to the allergen. As used herein, modification of the allergic response of an individual sensitive to a Bermuda grass pollen allergen can be defined as non-responsiveness or diminution in symptoms to the allergen, as determined by standard clinical procedures (See e.g., Varney et al., *British Medical Journal* 302: 265-269 (1990)). Initial screening for IgE binding to a protein allergen or fragments thereof may be performed by scratch tests or intradermal skin tests on laboratory animals or human volunteers, or in *in vitro* systems such as RAST (radioallergosorbent test), RAST inhibition, ELISA assay or radioimmunoassay (RIA).

Antigenic fragments of the present invention which have T cell stimulating activity, and comprise at least one T cell epitope are particularly desirable. T cell epitopes are believed to be involved in initiation and perpetuation of the immune response to a protein allergen which is responsible for the clinical symptoms of allergy. These T cell epitopes are thought to trigger early events at the level of the T helper cell by binding to an appropriate HLA molecule on the surface of an antigen presenting cell and stimulating the relevant T cell subpopulation. These events lead to T cell proliferation, lymphokine secretion, local inflammatory reactions, recruitment of additional immune cells to the site, and activation of the B cell cascade leading to production of antibodies. One isotype of these antibodies, IgE, is fundamentally important to the development of allergic symptoms and its production is influenced early in the cascade of events, at the level of the T helper cell, by the nature of the lymphokines secreted. A T cell epitope is the basic element or smallest unit of recognition by a T cell

receptor, where the epitope comprises amino acids essential to receptor recognition and may be contiguous and/or non-contiguous in the amino acid sequence of the protein. Amino acid sequences which mimic those of the T cell epitopes and which modify the allergic response to protein allergens are within the scope of this invention.

Exposure of patients to antigenic fragments of the present invention derived from protein allergens may tolerize or anergize appropriate T cell subpopulations such that they become unresponsive to the protein allergen and do not participate in stimulating an immune response upon such exposure. In addition, administration of an antigenic fragment of the present invention may modify the lymphokine secretion profile as compared with exposure to the naturally-occurring protein allergen or portion thereof (e.g., result in a decrease of IL-4 and/or an increase in IL-2). Furthermore, exposure to the antigenic fragment may influence T cell subpopulations which normally participate in the response to the allergen such that these T cells are drawn away from the site(s) of normal exposure to the allergen (e.g., nasal mucosa, skin, and lung) towards the site(s) of therapeutic administration of the fragment. This redistribution of T cell subpopulations may ameliorate or reduce the ability of an individual's immune system to stimulate the usual immune response at the site of normal exposure to the allergen, resulting in a diminution in allergic symptoms.

Bermuda grass protein allergens and fragments or portions derived therefrom (peptides) can be used in methods of diagnosing, treating and preventing allergic reactions to Bermuda grass pollen. Thus, the present invention provides therapeutic compositions comprising an isolated Bermuda grass pollen allergen or at least one fragment thereof and a pharmaceutically acceptable carrier or diluent. The Bermuda grass pollen allergen or at least one fragment thereof is preferably produced in a mast cell transformed to express the protein allergen or the fragment thereof or is synthetically prepared. Administration of the therapeutic compositions of the present invention to an individual to be desensitized can be carried out using known techniques. Bermuda grass pollen allergen or a fragment thereof can be administered to an individual in combination with, for example, an appropriate diluent, a carrier and/or an

adjuvant. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Pharmaceutically acceptable carriers include polyethylene glycol (Wie et al. (1981) *Int. Arch. Allergy Appl. Immunol.* 64:84-99) and liposomes (Strejan et al. (1984) *J. Neuroimmunol.* 7: 27). Such compositions will generally be administered by injection, oral administration, inhalation, transdermal application or rectal administration. The therapeutic compositions of the invention are administered to Bermuda grass pollen-sensitive individuals at dosages and for lengths of time effective to reduce sensitivity (i.e, reduce the allergic response) of the individual to Bermuda grass pollen. Effective amounts of the therapeutic compositions will vary according to factors such as the degree of sensitivity of the individual to Bermuda grass pollen, the age, sex, and weight of the individual, and the ability of the Bermuda grass pollen allergen or fragment thereof to elicit an antigenic response in the individual.

cDNA coding for a Bermuda grass pollen allergen (or the mRNA from which it was transcribed) or a portion thereof can be used to identify similar sequences in any variety or type of plant and thus, to identify or "pull out" sequences which have sufficient homology to hybridize to the cDNA of the protein allergen or mRNA or portion thereof. For example, cDNA of the present invention may hybridize to DNA from temperate grasses such as rye-grass, Kentucky Blue grass, Timothy grass and orchard grass, and from other grasses such as Bahia grass and sorghum, under conditions of low stringency. Those sequences which have sufficient homology (generally greater than 40%) can be selected for further assessment using the method described herein. Alternatively, high stringency conditions can be used. In this manner, DNA of the present invention can be used to identify, in other types of plants, preferably related families, genera, or species, sequences encoding polypeptides having amino acid sequences similar to that of a Bermuda grass pollen allergen, and thus to identify allergens in other species. Thus, the present invention includes not only Bermuda grass allergens, but also other allergens encoded by DNA which hybridizes to DNA of the present invention. The invention further includes protein allergens or fragments thereof which are immunologically related to a Bermuda grass protein allergen or fragments thereof, such as by antibody cross-reactivity, wherein the protein

allergens or fragments thereof are capable of binding to antibodies specific for the Bermuda grass pollen protein or fragments of the invention.

Proteins or peptides encoded by the cDNA of the present invention can be used, for example as "purified" allergens. Such purified allergens are useful in the standardization of allergen extracts which are key reagents for the diagnosis and treatment of sensitivity to a Bermuda grass pollen allergen. Furthermore, by using proteins or fragments thereof based on the nucleic acid sequences of clones B1, B2 and B4, anti-peptide antisera, polyclonal antibodies or monoclonal antibodies can be made using standard methods. These sera or polyclonal or monoclonal antibodies can be used to standardize allergen extracts and/or used in purification of native or recombinant protein allergens.

Through use of the Bermuda grass pollen proteins and antigenic fragments of the present invention, preparations of consistent, well-defined composition and biological activity can be made and administered for therapeutic purposes (e.g. to modify the allergic response of a Bermuda grass pollen-sensitive individual. Administration of such peptides or protein may, for example, modify B-cell response to Bermuda grass protein allergen, T cell response to a Bermuda grass protein allergen or both responses. Purified peptides can also be used to study the mechanism of immunotherapy of Bermuda grass pollen allergy and to design modified derivatives or analogues useful in immunotherapy.

It is possible to modify the structure of the protein allergens or fragments thereof of the invention, for such purposes as increasing solubility, enhancing therapeutic or preventive efficacy, or stability (e.g., shelf life *ex vivo*, and resistance to proteolytic degradation *in vivo*). A modified protein allergen or a modified fragment thereof can be produced in which the amino acid sequence has been altered, such as by amino acid substitution, deletion, or addition, to modify immunogenicity and/or reduce allergenicity, or to which a component has been added for the same purpose. For example, the amino acid residues essential to T cell epitope function can be determined using known techniques (e.g., substitution of each residue and determination of presence or absence of T cell reactivity). Those residues shown to be essential can be modified (e.g., replaced by another amino acid whose presence is shown to enhance T cell reactivity), as

can those which are not required for T cell reactivity (e.g., by being replaced by another amino acid whose incorporation enhances T cell reactivity but does not diminish binding to relevant MHC). In order to enhance stability and/or reactivity, a protein allergen or fragment thereof can also be modified to incorporate one or more polymorphisms in the amino acid sequence of a protein allergen resulting from natural allelic variation. Additionally, D-amino acids, non-natural amino acids or non-amino acid analogues can be substituted or added to produce a modified protein or fragment within the scope of this invention. Furthermore, protein allergens or fragments can be modified using the polyethylene glycol (PEG) method of A. Sehon and co-workers (Wie et al. *supra*) to produce a peptide conjugated with PEG. Modifications of protein allergens or fragments thereof can also include reduction/alkylation (Tarr in: *Methods of Protein Microcharacterization*, J.E. Silver ed. Humana Press, Clifton, NJ, pp 155-194 (1986)); acylation (Tarr, *supra*); esterification (Tarr, *supra*); chemical coupling to an appropriate carrier (Mishell and Shiigi, eds, *Selected Methods in Cellular Immunology*; WH Freeman, San Francisco, CA (1980); U.S. Patent 4,939,239); or mild formalin treatment (Marsh *International Archives of Allergy and Applied Immunology* 41: 199-215 (1971)).

Site-directed mutagenesis of DNA encoding a protein allergen or fragment thereof can be used to modify the structure. Such methods may involve PCR (Ho et al., *Gene* 77:51-59 (1989)) or total synthesis of mutated genes (Hostomsky, Z., et al., *Biochem. Biophys. Res. Comm.* 161:1056-1063 (1989)). To enhance bacterial expression, the aforementioned methods can be used in conjunction with other procedures to change the eucaryotic codons in DNA constructs encoding recombitope peptides to ones preferentially used in *E. coli*

Using the structural information now available, it is possible to design Bermuda grass protein allergen peptides which, when administered to a Bermuda grass pollen sensitive individual in sufficient quantities, will modify the individual's allergic response to Bermuda grass pollen. This can be done, for example, by examining the structure of a Bermuda grass pollen allergen or peptide such as those partially or fully encoded for by clones B1, B2 and B4, producing peptides (via an expression system, synthetically or otherwise) to be examined for their

ability to influence B cell and/or T cell responses in Bermuda grass pollen sensitive individuals and selecting appropriate B or T cell epitopes recognized by the cells. Protein, peptides or antibodies of the present invention can also be used for detecting and diagnosing sensitivity to Bermuda grass pollon allergens. For example, this could be done by combining blood or blood products obtained from an individual to be assessed for sensitivity to Bermuda grass pollen with an isolated antigenic fragment of a Bermuda grass pollen allergen, or isolated Bermuda grass pollen allergen, under conditions appropriate for binding of components (e.g., antibodies, T cells, B cells) in the blood with the fragment(s) or protein and determining the extent to which such binding occurs.

It is now also possible to design an agent or a drug capable of blocking or inhibiting the ability of Bermuda grass pollen allergen to induce an allergic reaction in Bermuda grass pollen sensitive individuals. Such agents could be designed, for example, in such a manner that they would bind to relevant anti-Bermuda grass protein allergen-IgE's, thus preventing IgE-allergen binding and subsequent mast cell degranulation. Alternatively, such agents could bind to cellular components of the immune system, resulting in suppression or desensitization of the allergic response to Bermuda grass pollen allergens. A non-restrictive example of this is the use of appropriate B and T cell epitope peptides, or modifications thereof, based on the cDNA/protein structures of the present invention to suppress the allergic response to Bermuda grass pollen. This can be carried out by defining the structures of B- and T cell epitope peptides which affect B and T cell function in *in vitro* studies with blood components from Bermuda grass pollen sensitive individuals.

The DNA used in any embodiment of this invention can be cDNA obtained as described herein, or alternatively, can be any oligodeoxynucleotide sequence having all or a portion of a sequence represented herein, or their functional equivalents. Such oligodeoxynucleotide sequences can be produced chemically or mechanically, using known techniques. A functional equivalent of an oligonucleotide sequence is one which is capable of hybridizing to a complementary oligonucleotide to which the sequence (or corresponding sequence portions) of Fig. 3, Fig. 5, or Fig. 8 or fragments thereof hybridizes, or the

sequence (or corresponding sequence portion) complementary to the nucleic acid sequences of Fig. 3, Fig. 5, or Fig. 8, and/or which encodes a product (e.g., a polypeptide or peptide) having the same functional characteristics of the product encoded by the sequence (or corresponding sequence portion) of Fig. 3, Fig. 5, or Fig. 8. Whether a functional equivalent must meet one or both criteria will depend on its use (e.g., if it is to be used only as an oligoprobe, it need meet only the first criterion and if it is to be used to produce a *Cynodon dactylon* allergen, it need only meet the second criterion).

This invention is further illustrated by the following non-limiting examples.

Example 1 Characterization and isolation of cDNA clones encoding Bermuda grass pollen allergens

Pollen of Bermuda grass, *Cynodon dactylon*, was purchased from Greer Laboratories, Lenoir, NC, USA. Bermuda grass inflorescence containing near mature anthers were collected in Melbourne and used as a source of fresh pollen.

Soluble proteins were extracted from rye-grass pollen by vigorous shaking in PBS (150 mM NaCl, pH 7.2) containing 1mM PMSF on ice for 3 hours. Conditions for electrophoresis and immunoblotting were essentially as described (Singh, M.B., and Knox, R.B., *Int. Archs. Allergy Appl. Immun.* 78: 300-304 (1985). For IgE antibody binding, blots were incubated in serum from an individual allergic to Bermuda grass pollen , diluted 1:5 in TBS/0.5% w/v BSA. The bound IgE was detected (Ford, D., and Baldo, B.A., *Int. Archs. Allergy Appl. Immun.* 81: 193-203 (1986), using ^{125}I-labelled anti-human IgE (Kallestad, USA).

Western blots were made from Bermuda grass pollen extract separated by SDS-PAGE and incubated in grass pollen -allergic patient's serum (Figure 1). The components in the pollen extract bind IgE, of which five bands show a particularly intense reaction. These are assumed to be major allergens. The five bands show apparent molecular weights of 17,30,32,45 and 55 kDA (Figure 1).

Poly (A^+) mRNA was isolated essentially as described (Herrin, D. and Michaels, F.B., *Plant Mol. Biol. Reporter* 2: 24-29 (1984)). cDNA was synthetized (Gubler, U. and Hoffman, B.J., *Gene* 25: 263-367 (1983)) and cloned into the EcoR1 site of the vector l-gtll. Immunological screening was done by plating the cDNA library, and screening duplicate filters with IgE from the serum of allergic individuals. Bound IgE was detected as above. The plaques which were IgE-positive on both of the duplicate filters were picked off and purified.

The cDNA library was screened with IgE antibodies from allergic patient's serum KR. The serum used was from a single patient (KR) who showed strong reactivity to Bermuda grass and rye-grass pollen samples. No patient's sera showed IgE binding only to Bermuda grass in the samples tested to date. Three clones were positive, B1, B2 and B4. Of these three clones, the fusion proteins of B1 and B4 bound IgE more strongly than B2 (Figure 2).

The sizes of the cDNA inserts of these clones were as follows: B1, 468 base pairs (bp); B2, 997bp; B4 429bp.

The cDNA clones B1, B2, and B4 were isolated from the phage, subcloned into pGEM-3Z vectors (Promega) and sequenced. DNA sequencing of the inserts of clones B1 and B4 was performed using T7 DNA polymerase and dideoxy nucleotide termination reactions (Sander et al., Proc. Natl. Acad. Sci. USA 74: 5460-5463, 1977). [^{35}S] dATP was used as the label (See Fig. 3 and Fig. 5). DNA sequencing of the EcoR1 insert in clone B2 was performed using standard dideoxy nucleotide termination reactions containing 7-deaza dGTP. 7-deaza dITP was used, if necessary, to resolve severe GC band compressions. [^{35}S]dATP was used as the label (See Fig. 8).

Example 2 Production of fusion protein from clone B1

The protein encoded by clone B1 was expressed as a fusion with b-galactosidase. Lambda-gt11 containing clone B1 cDNA inserts in *E. coli* were plated onto Luria-Bertani (LB) plates. After three hours growth at 42°C, the plates were overlayed with isopropyl-b-D-thiogalacto-pyranoside (IPTG) impregnated nitrocellulose filters (Hybond-C extra, Amersham) in order to

induce production of fusion protein. The plates were then incubated for four hours at 37°C, after which the filters were inverted and left for an additional four hours to enable the transfer of recombinant b-gal-B1 fusion protein to the nitrocellulose. These protein plaque lifts were incubated in 10% milk powder in phosphate buffered saline (PBS), to block unreacted binding sites.

Example 3 Production of pGEX-B2 fusion protein

The cDNA clone B2 was subcloned into the pGEX plasmid expression system. The expression of the protein as a fusion with glutathione S-transferase (GST) was carried out according to the procedure outlined by Smith and Johnson, *Gene* 67: 31-40 (1988). Overnight cultures of *E. coli* with recombinant pGEX-B2 plasmid were diluted 1:10 in fresh Luria broth and grown for 1 hour at 37°C with vigorous shaking. Fusion protein production was induced by adding IPTG to a final concentration of 0.1 mM. The cells were grown for a further 4-5 hours after which they were pelletted and resuspended in PBS (150mM NaCl, 16mM Na_2HPO_4, 4mM $NaHPO_4$, pH 7.2). The cells were lysed by subjecting the mixture to 3 freeze-thaw cycles in liquid nitrogen and the supernatant containing the GST-B2 fusion protein collected after centrifugation.

The supernatant containing the GST-B2 fusion protein was applied to the Superdex 75 HR 10\30 column (Pharmacia LKB, Sweden). The sample was eluted from the column with 50 mM PBS containing 0.02% sodium azide, at a constant flow rate of 1ml/min at room temperature (RT). The fractions containing the fusion protein were identified by dotting 5ml of each fraction onto nitrocellulose membrane (BA 0.45mm, Schleicher and Schuell, Dassel, Germany) and screening with sera from allergic patients for binding of specific IgE. The protein was concentrated using minicon Ultrafree-MC 10000NM WL filter unit (Millipore).

Example 4 Immunoblot Analysis and the production of polyclonal and monoclonal antibodies

Soluble proteins were extracted from Bermuda grass pollen by vigorous shaking in PBS (15mM NaCl, pH 7.2) on ice for 3 hours. Proteins were separated on 12% polyacrylamide gels using the Biorad mini Protean II system. The gels were either stained with Coomassie brilliant blue to visualize proteins or the proteins were transferred to nitrocellulose as according to the method described by Towbin *et al*, *Proc. Nat. Acad. Sci. USA*, 76: 4350,4354 (1979). Unreacted binding sites on these Western blots were blocked by incubation in 10% milk powder for at least one hour.

A. Screening with polyclonal antibodies.

Mouse antibodies specific for B2 antigens were prepared by immunizing female BALB/c mice with an intraperitoneal (i.p.) injection of 50mg of FPLC purified GST-B2 fusion protein in 0.1ml PBS and 0.1ml RIBI adjuvant. Fourteen days later a booster i.p. of the same material was given. After 10 days the mice were bled. The serum was screened for binding to dot blots of GST-B2 protein. Fourteen days later, the selected mice were given an i.p. booster of 0.2ml containing 50mg fusion protein only. Four days after the last immunization the mouse was bled. Serum was obtained by incubation at 37°C for 1 hour followed by centrifugation at 2500 rpm for 10 minutes. Polyclonal antibodies will be generated against the proteins or peptides partially or fully encoded by the nucleic acid sequences of clones B1 and B4 using the same method.

Western blots were incubated with the antibodies obtained above, for 3 hours at room temperature (RT). These blots were washed twice in PBS containing 0.1% Tween-20, twice in PBS, then incubated in peroxidase labelled-anti-mouse Ig for one hour at RT. This was followed by washing and color development as described by Singh, M.B. and Knox, R.B., *Int. Archs. Allergy Appl. Immun.* 78: 300-304 (1985). Results are shown in Fig. 10.

Figure 8 illustrates Western blots of Bermuda grass pollen proteins probed with (a) 1/100 dilution of clone B2 specific antiserum. (b) 1/250

dilution of clone B2 specific antiserum. (c) Serum of an allergic individual (d) soluble Bermuda grass pollen proteins separated by SDS-PAGE. The clone B2 specific antiserum recognizes a protein with MW of approximately 61kD. This protein binds IgE from serum of an allergic individual. Monoclonal antibodies will be raised against the proteins or peptides partially or fully encoded by the nucleic acid sequences of clones B1, B2 and B4. The spleen cells are being collected from mice selected as above four days after the boostering injection. B cells in the spleen will be fused with a suitable myeloma fusion partner using standard methodology (Harlow, E, and Lane D., *Antibodies. A Laboratory Manual.* Cold Spring Harbor Laboratory, New York (1988)).

B. Screening with human serum or affinity purified IgE

Six b-gal-B1 fusion protein plaque lifts and non-recombinant l-gt11 protein plaque lifts (as control) were prepared as described above. These protein plaque lifts were incubated overnight in serum from an individual allergic to Bermuda grass pollen, washed twice with PBS containing 0.1% Tween 20 and then twice with PBS only. The bound IgE antibodies were eluted with 0.1 M glycine-HCl, pH 2.6/1% BSA and used as affinity purified IgE to probe Western blots.

Western blots were incubated in sera from allergic individuals or in affinity purified IgE overnight at RT. IgE binding was detected by incubation of the blot in ^{125}I-labelled anti-human IgE overnight at RT and autoradiography, or by incubation in rabbit anti-human IgE followed by incubation in peroxidase labelled anti-rabbit Ig and color development as described above. Results are shown in Fig. 4.

Figure 4, columns b-d illustrate Western blots of Bermuda grass pollen proteins separated by SDS-PAGE and probed with (b) IgE affinity purified from non-recombinant Lambda-gt11. (c) IgE affinity purified from clone B1 fusion protein. (d) serum after incubation with B1 protein plaque lifts. (e) serum before incubation with B1 protein plaque lifts (total serum). After incubation of B1 protein plaque lifts in serum, IgE specific to a protein with MW 12kD is depleted from the serum. IgE affinity purified from B1 fusion

protein is specific for the same protein. This protein is indicated by the arrow.

Example 5 IgE Binding Studies with Recombinant B1, B2 and B4 clones

The sera of 12 patients that bound strongly to crude extracts of *Cynadon dactylon* pollen were tested for binding to recombinant B1, B2 and B4 clones expressed as a fusion protein. The results demonstrate that nine of 12 *C. dactylon* allergic patients that have antibodies binding to *C. dactylon* pollen crude extract on a Western blot of an SDS-PAGE gel have IgE antibody that binds to clones B1 and B4 as a fusion protein. Six of 12 *C. dactylon* allergic patients that have antibodies binding to *C. dactylon* pollen crude extract on a Western blot of an SDS-PAGE gel have IgE antibody that binds to clone B2 as a fusion protein. These results are shown in Table 1.

Table 1. Allergenicity of B1, B2, and B4 clones

ALLERGEN CLONE	IgE BINDING	% POSITIVE
B1	9/12	75%
B2	6/12	50%
B4	9/12	75%

Example 6 Cloning a full-length *Cyn d* allergen containing the sequence of B2 clone

Double-stranded cDNA is synthesized from approximately 4 mg pollen RNA using the cDNA Synthesis System Plus kit (BRL, Bethesda, MD). After a phenol extraction and ethanol precipitation, the cDNA is blunted with T4 DNA polymerase (Promega, Madison, WI), and ligated to ethanol precipitated, self-annealed, AT, 5' GGGTCTAGAGGTACCGTCCGATCGATCATT, and AL 5' p-AATGATCGATGCT, oligonucleotides for use in a modified Anchored PCR reaction *(Marsh et al., Allergen Nomenclature Bull. W.H.O* 64: 767-770 (1986); Roux and Dhanarajan, *Biotech.* 8: 48-57(1990); Rafnar et al., *J. Biol. Chem.* 266

linkered cDNA (5 ml) from a 20 ml reaction with 1 mg each of oligonucleotides AP, 5' GGGTCTAGAGGTACCGTCCG, and a primer CD-1, 5' TGGTCACGTTGGAGGAGT, which is based on B2 sequence. The primary polymerase chain reactions (PCR) is carried out in a programmable thermal controller from MJ Research, Inc. (Cambridge, MA) using the GeneAMP DNA Amplification kit (Perkin Elmer Cetus, Norwalk, CT) in a reaction containing 10 ml 10x buffer containing dNTPs mixed with 1 mg of each primer, cDNA, 0.5 ml Amplitaq DNA polymerase, and distilled water to 100 ml. Twenty-five rounds of amplification consisting of denaturation at 94°C for 1 minute, annealing of primers to the template at 60°C for 1.5 minutes, and chain elongation at 72°C for 2 minutes is carried out. Five percent (5 ml) of this primary amplification is then used in a secondary amplification with 1 mg each of CD-2, 5' GGGGATCCGGTGATGTCTTTGCACTT, a nested oligonucleotide primer based on known B2 sequence, and AP, as above. Oligonucleotide primers AP, AT and AL have been previously described (Rafnar et al., 1991, *supra*; Morgenstern et al., *Proc. Natl. Acad. Sci. USA* 88: 9690-9694; Griffith et al., *FEBS Lett.* 279 (199): 215 (1991); Rogers et al., *J. Immul.* 147: 2547-2552 (1991).

Amplified DNA is recovered by sequential chloroform, phenol, and chloroform extractions, followed by precipitation at -20°C with 0.5 volumes of 7.5 ammonium acetate and 1.5 volumes of isopropanol. After precipitation and washing with 70% ethanol, the DNA is simultaneously digested with *Xbal* and *Bam* HI in a 15 ml reaction and electrophoresed through a preparative 3% SeaPlaque low melt agarose (FMC Corp., Rockland, ME). The appropriate sized DNA band is visualized by ethidium bromide (EtBr) staining, excised, and ligated into appropriately digested M13mp19 dideoxy DNA sequencing (Sanger et al., *supra)* with the Sequenase kit (U.S. Biochemicals, Cleveland, OH).

Full-length cDNA sequences comprising the cDNA sequences of clones B1 and B4 which full-length sequences encode Bermuda grass pollen protein allergens are being elucidated using PCR methodology as described for clone B2 above. Nested oligonucleotide primers based on the non-coding strand sequence complementary to the nucleotide sequences given in Figs. 3 and 5 will be used with oligonucleotide primer AP to amplify full-length clones from AT/AL

linkered cDNA derived from *Cynodon dactylon* RNA.

Alternatively, double stranded or single stranded probes will be prepared from all or a portion of the nucleotide sequence of clones B1, B2 or B4 (see Figs. 3, 5, and 8). These probes will be used to probe a library, for example a lgt10 or lgt11 library, to identify homologous clones. Selected clones will be sequenced as described to identify full-length clones for B1, B2 and B4. Specific probes will be generated and the libraries will be screened using standard procedures (Smart et al., *supra*).

Although the invention has been described with reference to its preferred embodiments, other embodiments can achieve the same results. Variation and modifications to the present invention will be obvious to those skilled in the art and it is intended to cover in the appended claims all such modifications and equivalents that follow in the true spirit and scope of this invention.

CLAIMS

1. A nucleic acid sequence coding for a protein allergen of the species *Cynodon dactylon*, or at least one antigenic fragment thereof or the functional equivalent of said nucleic acid sequence.

2. The nucleic acid sequence of claim 1 comprising a nucleic acid sequence selected from the group consisting of the nucleic acid sequence of Clone B1 as shown in Fig. 3, the nucleic acid sequence of Clone B4 as shown in Fig. 5, and the nucleic acid sequence of Clone B2 as shown in Fig. 8.

3. An expression vector comprising the nucleic acid sequence of claim 1.

4. An expression vector of claim 3 wherein said nucleic acid sequence is selected from the group consisting of the nucleic acid sequence of Clone B1 as shown in Fig. 3, the nucleic acid sequence of Clone B4 as shown in Fig. 5, and the nucleic acid sequence of Clone B2 as shown in Fig. 8.

5. A host cell transformed to express a protein or peptide encoded by the nucleic acid sequence of claim 1.

6. A host cell transformed to express a protein or peptide encoded by the nucleic acid sequence of claim 2.

7. An isolated protein allergen of the species *Cynodon dactylon* or at least one antigenic fragment thereof.

8. The isolated protein allergen or antigenic fragment of claim 7 produced in a host cell transformed with the nucleic acid sequence of claim 1.

9. The isolated protein allergen or antigenic fragment of claim 7, produced in a host cell transformed with the nucleic acid sequence of claim 2.

10. A method of producing a protein allergen of the species *Cynodon dactylon* or at least one antigenic fragment thereof comprising, culturing a host cell transformed with a nucleic acid sequence encoding a protein allergen of the species *Cynodon dactylon* or antigenic fragment thereof in an appropriate medium to produce a mixture of cells and medium containing said protein allergen or antigenic fragment thereof; and purifying said mixture to produce substantially pure protein allergen of the species *Cynodon dactylon* or fragment thereof.

11. An isolated protein allergen of the species *Cynodon dactylon* comprising an amino acid sequence selected from the group consisting of the amino acid sequence of clone B1 as shown in Fig. 3, the amino acid sequence of clone B4 as shown in Fig. 5, and the amino acid sequence of clone B2 as shown in Fig. 8, or at least one isolated antigenic fragment thereof.

12. An antigenic fragment of claim 7 which has T cell stimulating activity.

13. An antigenic fragment of claim 11 which has T cell stimulating activity.

14. An antigenic fragment of claim 13 which has minimal immunoglobulin E stimulating activity.

15. An antigenic fragment of claim 13 which does not bind immunoglobulin E specific for a protein allergen of the species *Cynodon dactylon* or if binding of the fragment to said immunoglobulin E occurs, such binding does not result in histamine release from mast cells or basophils.

16. An isolated antigenic fragment of claim 13 which binds immunoglobulin E to a substantially lesser extent than the protein allergen from which the fragment is derived binds said immunoglobulin E.

17. An isolated protein allergen or antigenic fragment of claim 7 which modifies, in a Bermuda grass pollen-sensitive individual to which it is administered, the allergic response of the individual to a Bermuda grass pollen allergen.

18. An isolated protein allergen or antigenic fragment of claim 11 which modifies, in a Bermuda grass pollen-sensitive individual to which it is administered, the allergic response of the individual to a Bermuda grass pollen allergen.

19. An isolated protein allergen or antigenic fragment of claim 17 which modifies the B cell response of the individual to a Bermuda grass pollen allergen, the T cell response of the individual to a Bermuda grass pollen allergen, or both the B cell response and the T cell response of the individual to a Bermuda grass pollen allergen.

20. An isolated protein allergen or antigenic fragment of claim 18 which modifies the B cell response of the individual to a Bermuda grass pollen allergen, the T cell response of the individual to a Bermuda grass pollen allergen, or both the B cell response and the T cell response of the individual to a Bermuda grass pollen allergen.

21. A modified protein allergen of Bermuda grass pollen which, when administered to a Bermuda grass pollen sensitive individual, reduces the allergic response of the individual to a Bermuda grass pollen allergen.

22. A modified protein allergen of claim 21 wherein the protein allergen that is modified comprises an amino acid sequence selected from the group consisting of the amino acid sequence of clone B1 as shown in Fig. 3, the amino acid sequence encoded by clone B4 as shown in Fig. 5, and the amino acid sequence encoded by clone B2 as shown in Fig. 8.

23. At least one modified fragment of a protein allergen of Bermuda grass pollen which, when administered to a Bermuda grass pollen-sensitive individual, reduces the allergic response encoded by the individual to said protein allergen.

24. At least one modified fragment of a protein allergen of claim 25 wherein said protein allergen comprises an amino acid sequence selected from the group consisting of the amino acid sequence encoded by clone B1 as shown in Fig. 3, the amino acid sequence encoded by clone B4 as shown in Fig. 5, and the amino acid sequence encoded by clone B2 as shown in Fig. 8.

25. An isolated protein allergen or antigenic fragment thereof which is immunologically cross-reactive with a protein allergen of Bermuda grass pollen, said protein allergen of Bermuda grass pollen comprising an amino acid sequence selected from the group consisting of the amino acid sequence encoded by clone B1 as shown in Fig. 3, the amino acid sequence encoded by clone B4 as shown in Fig. 5, and the amino acid sequence encoded by clone B2 as shown in Fig. 8.

26. A therapeutic composition comprising a protein allergen of Bermuda grass pollen, or at least one fragment thereof and a pharmaceutically acceptable carrier or diluent.

27. A therapeutic composition of claim 26 wherein the protein allergen comprises an amino acid sequence selected from the group consisting of the amino acid sequence encoded by clone B1 as shown in Fig. 3, the amino acid sequence encoded by clone B4 as shown in Fig. 5, and the amino acid sequence

encoded by clone B2 as shown in Fig. 8.

28. A method of treating sensitivity in an individual to a protein allergen of Bermuda grass pollen, comprising administering to said individual a therapeutically effective amount of a therapeutic composition of claim 26.

29. A method of treating sensitivity to in an individual to Bermuda grass pollen, comprising administering to said individual a therapeutically effective amount of a therapeutic composition of claim 27.

30. A method of detecting in an individual sensitivity to a protein allergen of Bermuda grass pollen, comprising combining a blood sample obtained from said individual with an isolated protein allergen of Bermuda grass pollen or antigenic fragment thereof produced in a host cell transformed with the nucleic acid sequence of claim 1, or chemically synthesized under conditions appropriate for binding of blood components with the protein allergen or fragment thereof and determining the extent to which such binding occurs.

31. The method of claim 30 wherein the extent to which binding occurs is determined by assessing T cell function, T cell proliferation, B cell function, binding of the protein or fragment thereof to antibodies present in the blood or a combination thereof.

32. A method of detecting in an individual sensitivity to a protein allergen of Bermuda grass pollen, comprising combining a blood sample obtained from said individual with an isolated protein allergen of Bermuda grass pollen or antigenic fragment thereof produced in a host cell transformed with the nucleic acid sequence of claim 2, or chemically synthesized under conditions appropriate for binding of blood components with the protein allergen or fragment thereof and determining the extent to which such binding occurs.

33. The method of claim 32 wherein the extent to which binding occurs is determined by assessing T cell function, T cell proliferation, B cell function, binding of the protein or fragment thereof to antibodies present in the blood or a combination thereof.

34. Monoclonal antibodies specifically reactive with a Bermuda grass pollen allergen or an antigenic fragment thereof.

35. Polyclonal antibodies specifically reactive with a Bermuda grass pollen allergen or an antigenic fragment thereof.

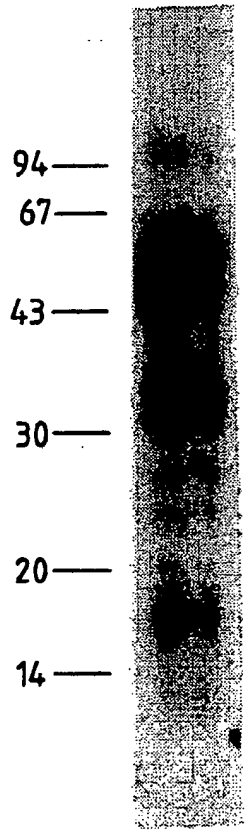


Fig. 1

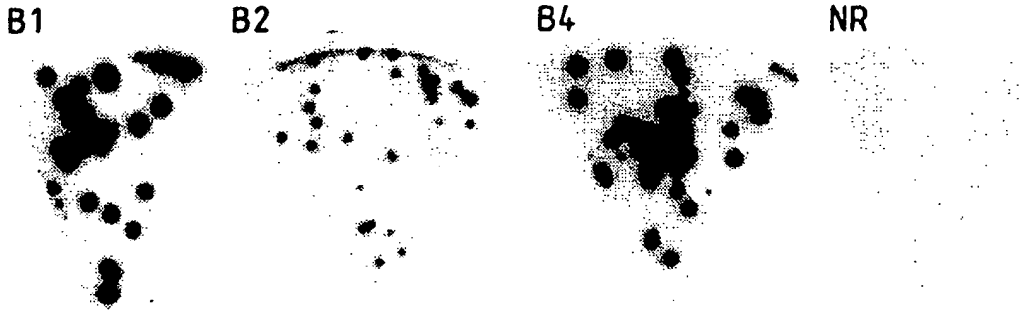


Fig. 2

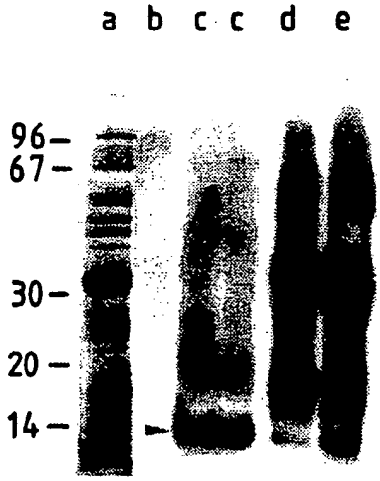
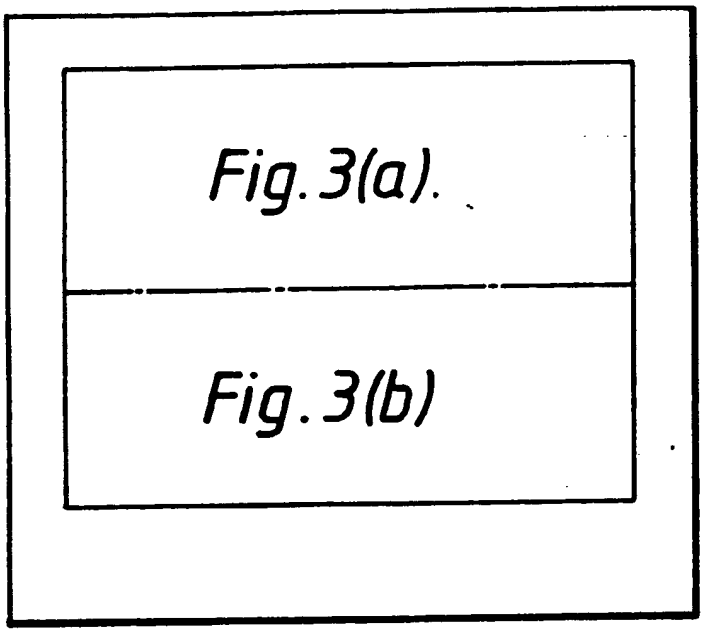


Fig. 4



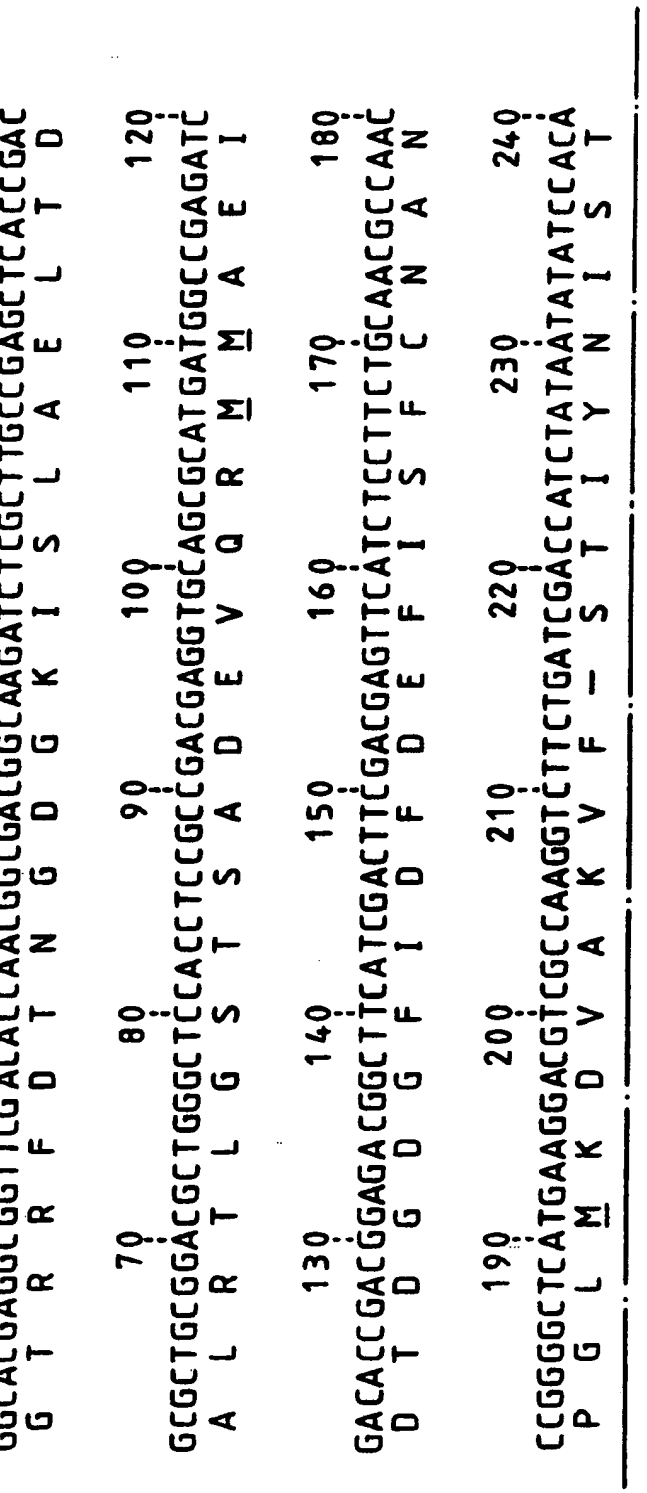
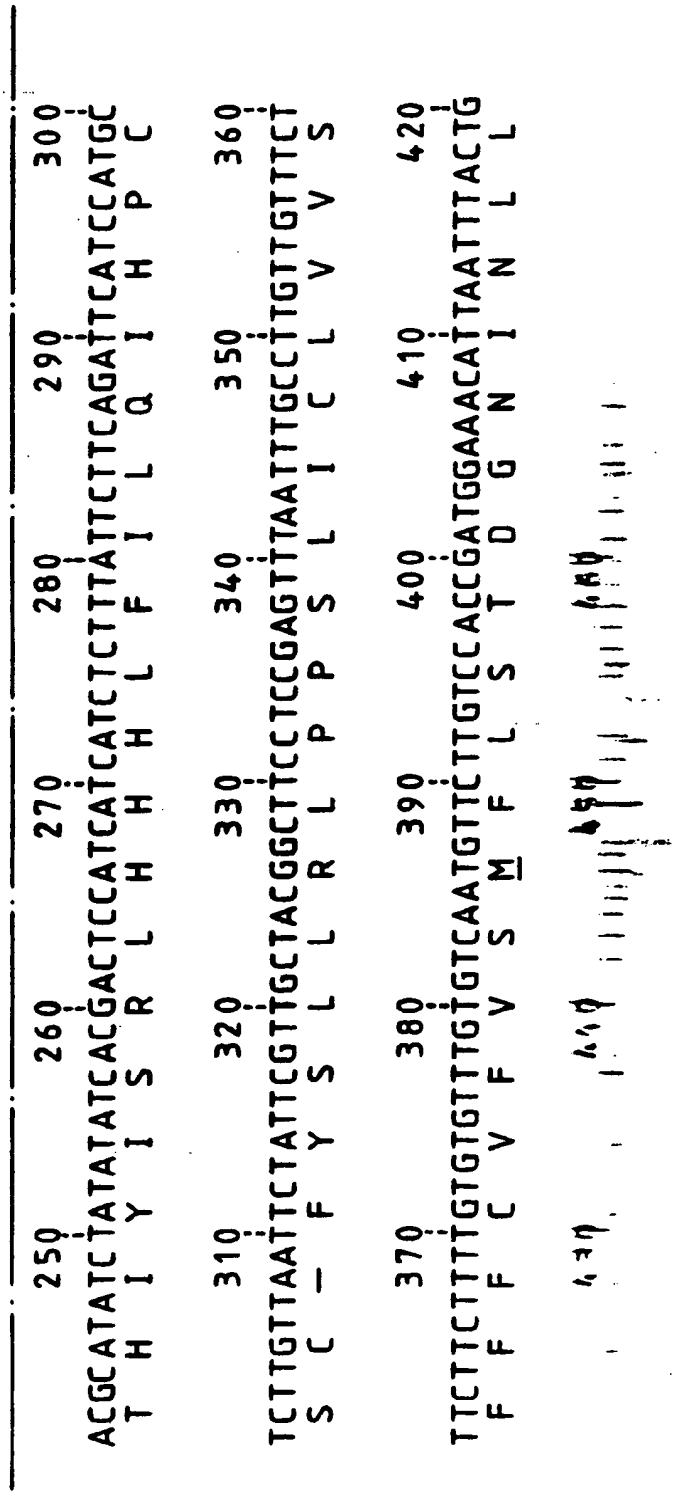


Fig.3(a).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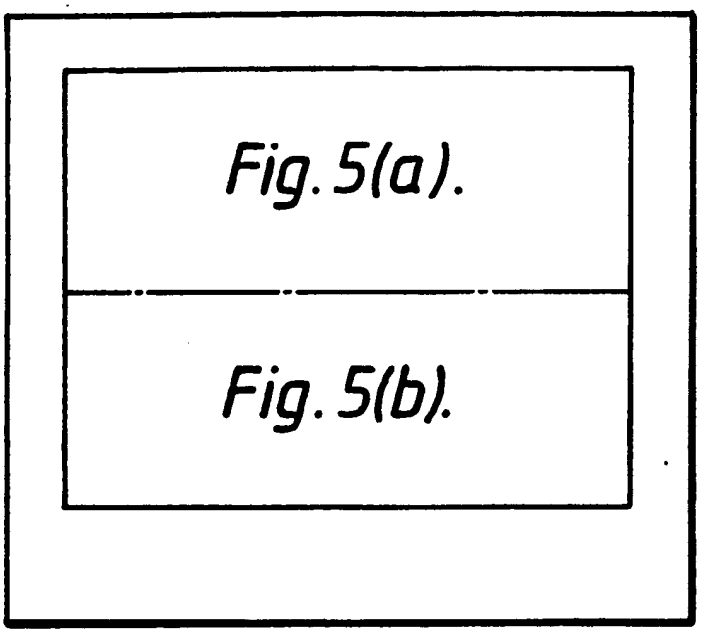
Fig. 5(a).
Fig. 5(b).

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  G  T  S  F  K  K  R  F  D  T  N  G  D  G  K  I  S  L  A  E  L

                70        80        90        100       110       120
ACCGACGCGCTGCGGACGCTGGGCTCCACCTCCGCCGACGAGGTGCAGCGCATGATGGCC
  T  D  A  L  R  T  L  G  S  T  S  A  D  E  V  Q  R  M  M  A

                130       140       150       160       170       180
GAGATCGACACCGACGGAGACGGCTTCATCGACTTCGACGAGTTCATCTCCTTCTGCAAC
  E  I  D  T  D  G  D  G  F  I  D  F  D  E  F  I  S  F  C  N

                190       200       210       220       230       240
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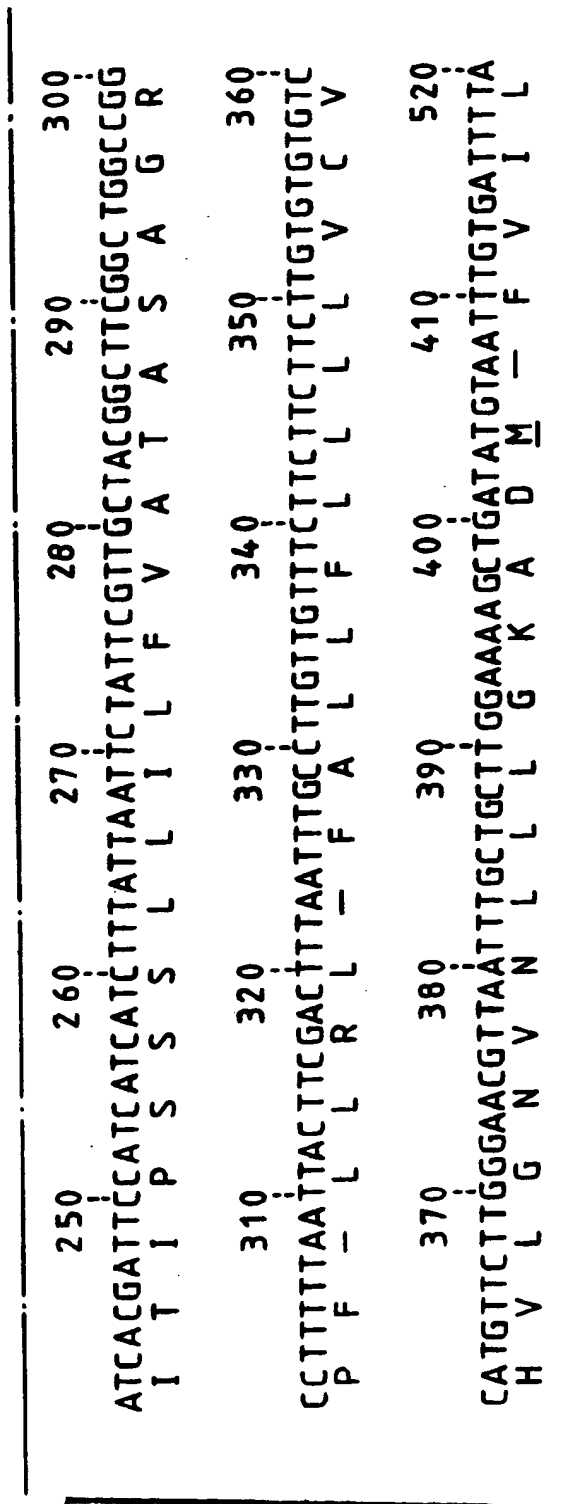
Fig.5(a).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T R A

Fig.5.(b).

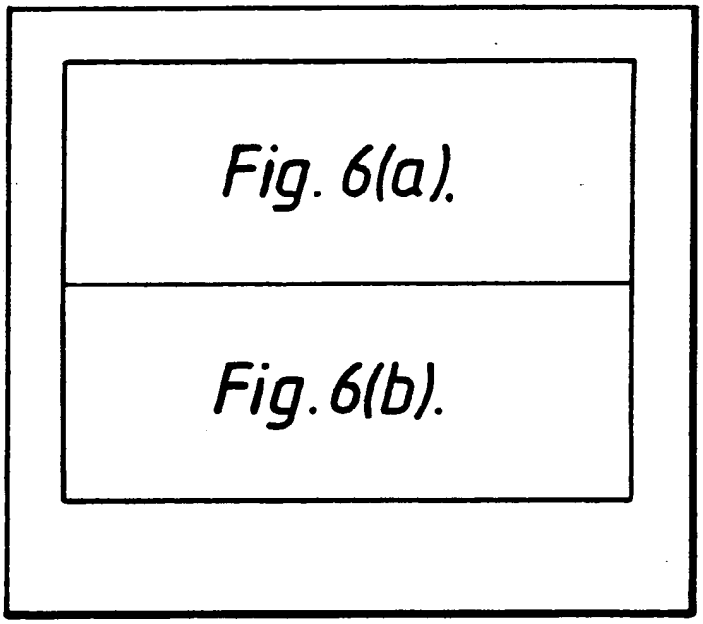
Fig. 6(a).
Fig. 6(b).

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CYND$B1  - GAATTCGGCACGAG------GCGGTTCGACACCAACGGCGACGGCAAGAT -44
CYND$B4  - GAATTCGGCACGAGCTTCAAGCGCTTCGACACCAACGGCGACGGCAAGAT -50

CYND$B1  - CTCGCTTGCCGAGCTCACCGACGCGCTGCGGACGCTGGGCTCCACCTCCG -94
CYND$B4  - CTCGCTTGCCGAGCTCACCGACGCGCTGCGGACGCTGGGCTCCACCTCCG -100

CYND$B1  - CCGACGAGGTGCAGCGCATGATGGCCGAGATCGACACCGACGGACACGGC -144
CYND$B4  - CCGACGAGGTGCAGCGCATGATGGCCGAGATCGACACCGACGGACACGGC -150

CYND$B1  - TTCATCGACTTCGACGAGTTCATCTCCTTCTGCAACGCCAACCCGGGGCT -194
CYND$B4  - TTCATCGACTTCGACGAGTTCATCTCCTTCTGCAACGCCAACCCGGGGCT -200

CYND$B1  - CATGAAGGACGTCGCCAAGGTCTTCTGATCGACCATCTATAATATCCA -244
CYND$B4  - CATGAAGGACGTCGCCAAGGTCTTCTGATCGACCATCTAC------TC-- -242
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Fig.6.(a)

```
CYND$B1   - CAACGCATATCTATATATCACGACTCCATCATCATCTCTTTATTCTTCAG -294
CYND$B4   - --------ATCTAT----CACGATTCCATCATCATCT--TTATTAATTCT -278

CYND$B1   - ATTCATCCATGCTCTTGTTAATTCTATTCGTTGCTACGGCTTCCTCCGAG -344
CYND$B4   - ATTCGT---TGCTACGGCTTCGGCTGGCCGGCCTTTTTAATTACTTCGAC -325

CYND$B1   - TTTAATTTGCCTTGTTGTTTCTTTCTTCTTTTGTGTGTTTGTGTCAATGT -394
CYND$B4   - TTTAATTTGCCTTGTTGTTTCTT-CTTCTTCT-TGTGT--GTGTCCATGT -371

CYND$B1   - TCTTGTCCACCGATGGAAACATTAATTTACTGCTTGGAAAAGCTGATATG -444
CYND$B4   - TCTTG----------GGAACGTTAATTTGCTGCTTGGAAAAGCTGATATG -411

CYND$B1   - TAATTTGTGATTTTAAGTTCCGCTCGTGCCGAATTC -480
CYND$B4   - TAATTTGTGATTTTAA------CTCGTGCCGAATTC -441
```

Fig.6.(b).

```
CYND$B1_1  -  GT--RRFDTNGDGKISLAELTDALRTLGSTSADEVQRMMAEIDTDGDGFI  -48
              ---   s  ---------------------------------------
CYND$B4_1  -  GTSFKRFDTNGDGKISLAELTDALRTLGSTSADEVQRMMAEIDTDGDGFI  -50

CYND$B1_1  -  DFDEFISFCNANPGLMKDVAKVF  -71
              -----------------------
CYND$B4_1  -  DFDEFISFCNANPGLMKDVAKVF  -73
```

Fig.7.

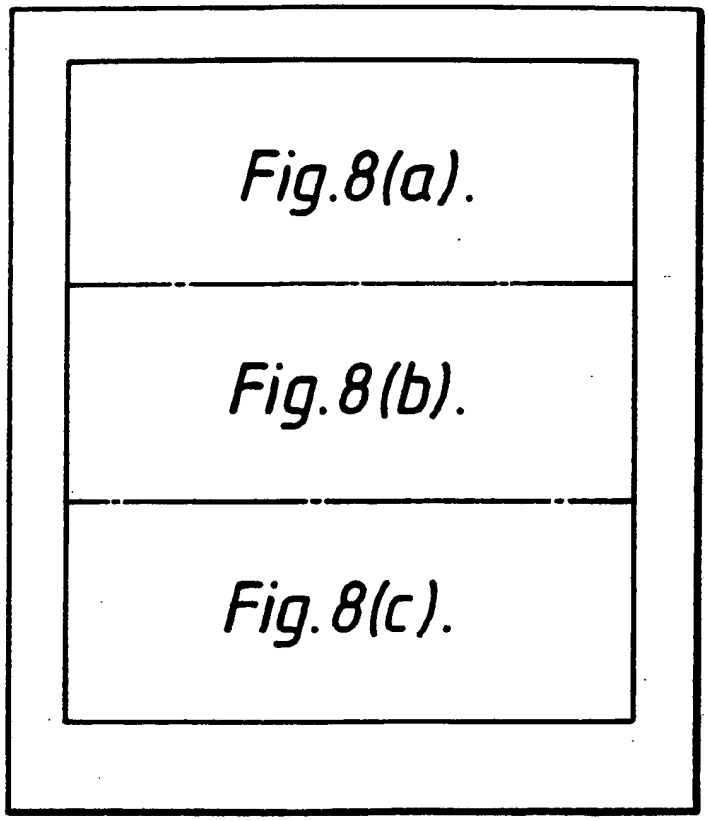
Fig.8(a).
Fig.8(b).
Fig.8(c).

```
        10         20         30         40         50         60
GGCACGAGGAACAATGCCACTGTCTCCGGCATCAAGCTCCTCAACTCGAAATTCTTCCAC
 G  T  R  N  N  A  T  V  S  G  I  K  L  L  N  S  K  F  F  H

        70         80         90        100        110        120
ATCAACATCGACAAGTGCAAAGACATCACCGTCAAGGACGTCACAATCACGGCGCCTGGG
 I  N  I  D  K  C  K  D  I  T  V  K  D  V  T  I  T  A  P  G

       130        140        150        160        170        180
GACGTCGAGAACACAGACGGCGTCCACGTCGGCGACTCCTCCAACGTGACCATCACCAAC
 D  V  E  N  T  D  G  V  H  V  G  D  S  S  N  V  T  I  T  N

       190        200        210        220        230        240
TCGAGCATCGGCACCGGCGATGACTGCGTCTCCGTCGGGTCAGGGAGCGCCGGCGTCATG
 S  S  I  G  T  G  D  D  C  V  S  V  G  S  G  S  A  G  V  M

       250        260        270        280        290        300
GTTACGGGGATCACCTGCGGCCCCGGACAGGGCATCAGCGTTGGTTGCCTCGGCCGGTAC
 V  T  G  I  T  C  G  P  G  Q  G  I  S  V  G  C  L  G  R  Y

       310        320        330        340        350        360
AAGGACGAGAAGGACGTGAGCGACGTCACCGTGCGCGACTGCGTGCTCAAGAGCACCACC
 K  D  E  K  D  V  S  D  V  T  V  R  D  C  V  L  K  S  T  T
```

Fig. 8.(a).

```
        370         380         390         400         410         420
AATGGCGTGCGCATCAAGTCCTACGAGGACGCCGAGTCCGTCATCACTGCCTCCAGGCTC
 N  G  V  R  I  K  S  Y  E  D  A  E  S  V  I  T  A  S  R  L

        430         440         450         460         470         480
ACCTTCGAGAACATCAGGATGGAAGACGTGGCCAAGCCCATCATCATCGACCAGTACTAC
 T  F  E  N  I  R  M  E  D  V  A  K  P  I  I  I  D  Q  Y  Y

        490         500         510         520         530         540
TGCCCAGAGAAGGTGTGCCCGGGCAAGAAGAGCAGCACCTCCCACGTCGCCGTCAAGGAC
 C  P  E  K  V  C  P  G  K  K  S  S  T  S  H  V  A  V  K  D

        550         560         570         580         590         600
GTCGTGTTCCGCAACATCACCGGAACGTCGTCCACGCCTGAGGCCGTCAGCCTGCTCTGC
 V  V  F  R  N  I  T  G  T  S  S  T  P  E  A  V  S  L  L  C

        610         620         630         640         650         660
TCCGAGACCCAGCCATGCAGTGGCGTCGAGCTCATCGACGTCAAGGTGGAGTACGCAGGC
 S  E  T  Q  P  C  S  G  V  E  L  I  D  V  K  V  E  Y  A  G

        670         680         690         700         710         720
AAGAACAACAAGACCATGGCAGTGTGCACCAACGCCAAAGGGGTCGCCAAGGGCAGCATC
 K  N  N  K  T  M  A  V  C  T  N  A  K  G  V  A  K  G  S  I
```

Fig.8.(b).

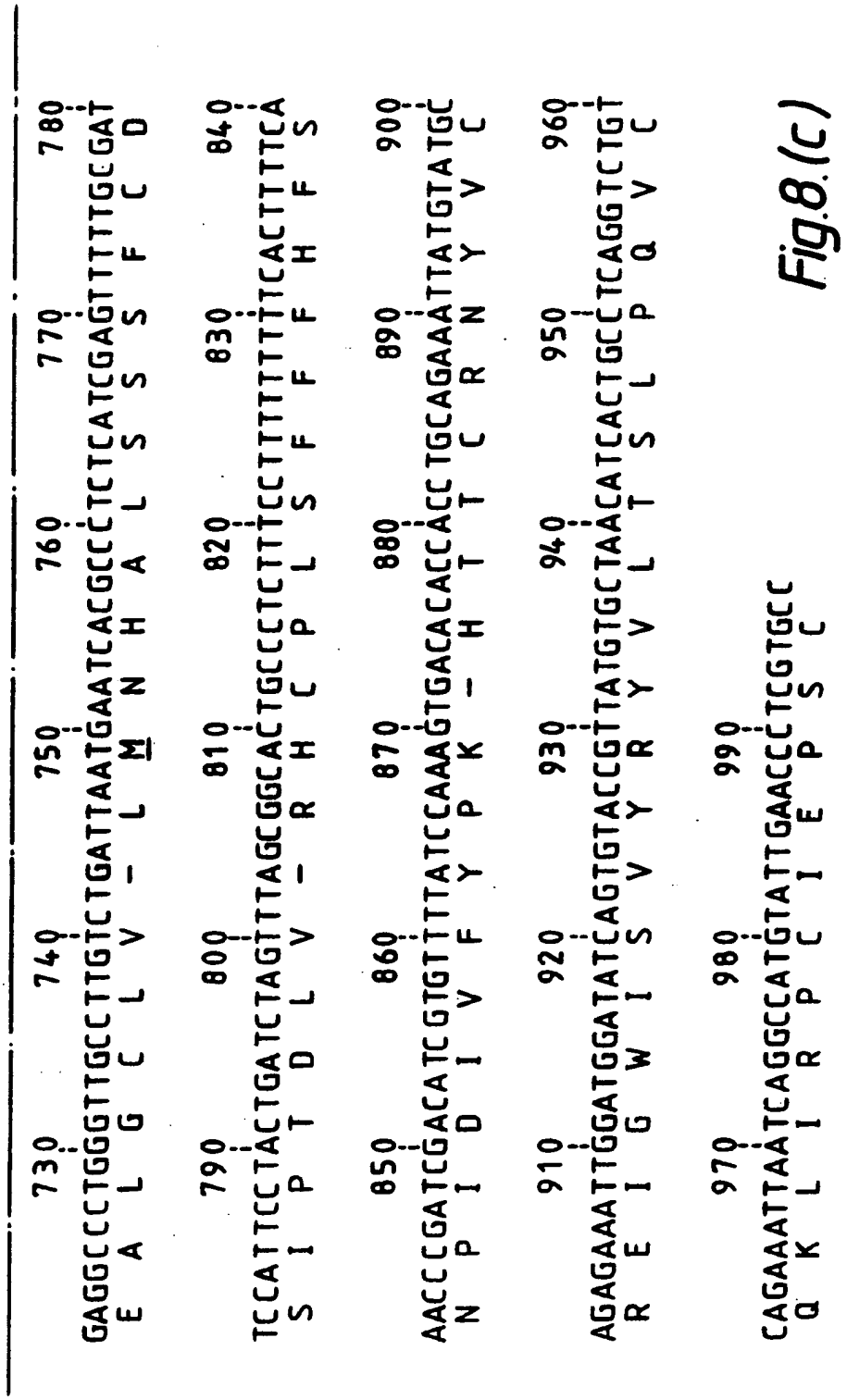
730 740 750 760 770 780
GAGGCCCTGGGTTGCCTTGTCTGATTAATGAATCACGCCCTCTCATCGAGTTTTTGCGAT
E A L G C L V - L M N H A L S S S F C D
790 800 810 820 830 840
TCCATTCCTACTGATCTAGTTTAGCGGCACTGCCCTCTTTCCTTTTTTTTTCACTTTTCA
S I P T D L V - R H C P L S F F F H F S
850 860 870 880 890 900
AACCCGATCGACATCGTGTTTTATCCAAAGTGACACACCACCTGCAGAAATTATGTATGC
N P I D I V F Y P K - H T T C R N Y Y V C
910 920 930 940 950 960
AGAGAAATTGGATGGATATCAGTGTACCGTTATGTGCTAACATCACTGCCTCAGGTCTGT
R E I G W I S V Y R Y Y V L T S L P Q V C
970 980 990
CAGAAATTAATCAGGCCATGTATTGAACCCTCGTGCC
Q K L I R P C I E P S C

Fig.8.(c)

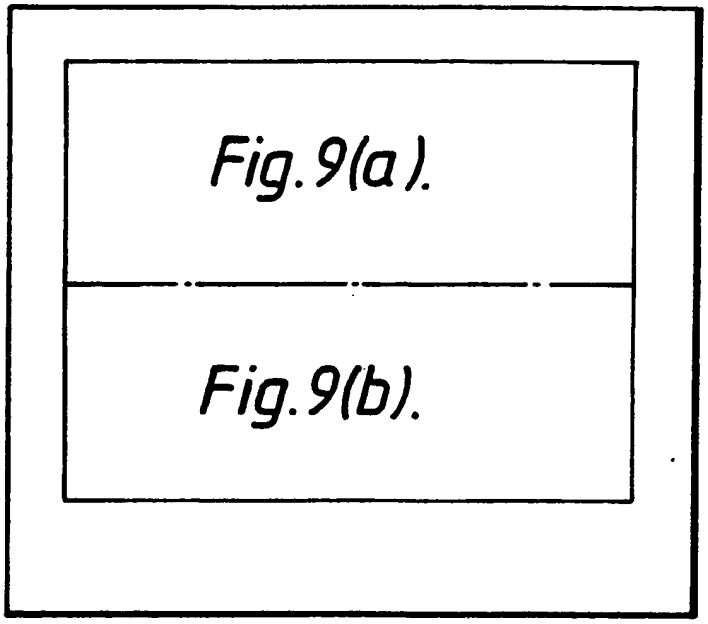
Fig. 9(a).
Fig. 9(b).

SUBSTITUTE SHEET

```
PGLR$LYCES- MVIQRNSILLLIIIFASSISTCRSNVIDDNLFKQVYDNILEQEFAHDFQA -50
CYND$B2   -                                        GTRNNATV------- -8
                                                     ss
PGLR$LYCES- YLSYLSKNIESNNNIDKVDKNGIKVINVLSFGAKGDGKTYDNIAFEQAWN -100
CYND$B2   - ------------------------------------------------- -8
PGLR$LYCES- EACSSRTPVQFVVPKNKNYLLKQITFSGPCRSSISVKIFGSLEASSKISD -150
CYND$B2   - ------------------------------------------------- -8
PGLR$LYCES- YKDRRLWIAFDSVQNLVVGGGGTINGNGQVWWPSSCKINKSLPCRDAPTA -200
CYND$B2   - -----------SGIKLLNSKFFHINIDKCKDITVKDVTITAPGDVENTDG -47
                       s  s  s  s  s  s  s
PGLR$LYCES- LTFWNCKNLKVNNLKSKNAQQIHIKFESCTNVVASNLMINASAKSPNTDG -250
CYND$B2   - VHVGDSSNVTITNSSIGTGDDCVSVGSGSAGVMVTGITCGPGQGISVGCL -97
             s  s  s  s  s  s  s  s  s  s  s  s
PGLR$LYCES- VHVSNTQYIQISDTIIGTGDDCISIVSGSQNVQATNITCGPGHGISIGSL -300
```

Fig.9(a).

SUBSTITUTE SHEET

```
CYND$B2    - GRYKDEKDVSDVTVRDCVLKSTTNGVRIKSYEDAESVITASRLTFENIRM -147
PGLR$LYCES- GSGNSEAYVSNVTVNEAKIIGAENGVRIKTWQGGSG--QASNIKFLNVEM -348
CYND$B2    - EDVAKPIIIDQYYCP----------EKVCPGKKSSTSHVAVKDVVFRNI -186
PGLR$LYCES- QDVKYPIIIDQNYCDRVEPCIQQFSA----------VQVKNVVYENI -385
CYND$B2    - TGTSSTP------------------------------------------ -193
PGLR$LYCES- KGTSATKVAIKFDCSTNFPCEGIIMENINLVGESGKPSEATCKNVHFNNA -435
CYND$B2    - ----------EAVSLLCSETQPCSGVELIDVKVEYAGKNNKTMAVCT -230
PGLR$LYCES- EHVTPHCTSLEISEDEALLYN---------------------------- -456
CYND$B2    - NAKGVAKGSIEALGCLV -247
PGLR$LYCES- ----------------Y -457
```

Fig.9(b).

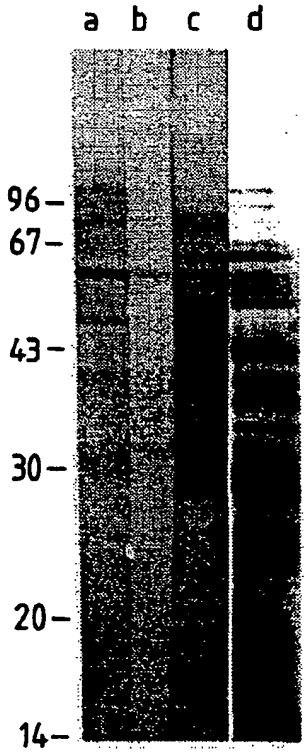

Fig. 10

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent classification (IPC) or to both National Classification and IPC

Int. Cl.[5] C07K 7/10, 13/00, 15/10, A61K 39/36, C12N 15/29, 15/62, C12P 21/08, G01N 33/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C07K 7/10, 13/00, 15/10, C12N 15/29, 15/62
CHEMICAL ABSTRACTS	(CAS 82) keywords (CYNODON OR BERMUDA(I)GRASS) AND POLLEN: AND (ANTIGEN: or ALLERGEN:),
CHEMICAL ABSTRACTS	STN CAS-ON LINE PROTEIN SEQUENCE SEARCH

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU: IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category*	Citation of Document, [11] with indication, where appropriate of the relevant passages [12]	Relevant to Claim No [13]
X	S.A. Medical Journal, vol 51 (1977), A. Orren and L. B. Dowdle, "Studies on Bermuda grass Pollen Allergens", pages 586-591, (see whole document).	7
X	J. Allergy Clin. Immunol., vol 81 (1988), F. Matthiesen et al. "Charcterization of the major allergen of Cynodon Dactylon (Bermuda grass) Pollen", page 266, abstract No. 393, (see whole abstract).	7,34,35
X	Clinical Allergy, vol 18 (1988), "Identification of allergens and antigens of Bermuda grass (Cynodon dactylon) pollen by immunoblot analysis", pages 401-409, (see whole document). (continued)	7, 35

* Special categories of cited documents : [10]

"A" Document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior t the international filing date but later than the priority date claimed

"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18 June 1992 (18.06.92)	26 June 1992 (26.06.92)
International Searching Authority	Signature of Authorized Officer
AUSTRALIAN PATENT OFFICE	T. SUMMERS

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	US,A,4681752 (Melillo) 21 July 1987 (21.07.87). See column 2 line 56, table II and the claims.	26
X	Chemical Abstracts, vol. 107, no 13, issued 1987, September 28, (Columbus, Ohio, U.S.A.) S.N. Su et al., "Isolation and partial characterization of allergen from Bermuda grass pollen", page 485, column 2 abstract 113940c, (Chung-hua Min Kuo Wei Sheng Wu Chi Mien I Hsueh Tsa Chih, 1986 19(4) pages 263-75 (chinese)) (whole abstract).	7, 35
X	J. Allergy Clin. Immunol. vol 79, no 5 (May 1987), S.A. Ford and B.A. Baldo, "Identification of Bermuda grass (Cynodon dactylon) pollen allergens by electroblotting", pages 711-720 (see whole document).	7, 35

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4a

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant, Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, [11] with indication, where appropriate of the relevant passages [12]	Relevant to Claim No [13]
X	Allergy, vol 46, 1991, Z.N. Chang et al., "Analysis of allergenic components of Bermuda grass pollen by monoclonal antibodies", pages 520-528 (see whole document).	7, 17, 26, 34, 35
X	EP,A2,192321 (BEECHAM GROUP PLC) 27 August 1986, (27.08.86). (see claim 7 and pages 8 and 9).	26
X	Electrophoresis, vol.8, 1987, E. R. Tovey and B. A. Baldo, "Characterization of allergens by protein blotting" pages 452-463, (see page 455 column 1 and figure 1).	7, 35

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL APPLICATION NO. PCT/AU 92/00108

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member		
US 4681752	CA 1207663	CH 650925	DK 2397/82
	EP 67130	FR 2512670	GB 2099699
	IL 65875	JP 57212114	NO 821832
EP 192321	AU 52455/86	DK 249/86	GB 8501400
	JP 61172829	NZ 214853	PT 81867
	ZA 8600355		